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Androgen-induced Differentiation of the Fibrocartilage of Os Penis Cultured *in vitro*

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ABSTRACT—Primordia of the fibrocartilage of os penis of the rat were cultured in vitro in the presence of testosterone, and the effects of testosterone on the expression of type I collagen, type II collagen, vimentin, the activity of alkaline phosphatase (ALP), and Alcian blue-stainable extracellular matrix (ECM) were examined histochemically. Explants cultured in the presence of testosterone formed fibrocartilage while control explants cultured without testosterone formed fibroblastic tissue. Type II collagen, ALP, and Alcian blue stainable ECM were detected only in the explants that had been cultured with testosterone. Calcification of the fibrocartilage was observed in explants that had been cultured for 21 days. By contrast, levels of type I collagen and vimentin increased in explants cultured with or without testosterone. These results demonstrate that testosterone induces terminal differentiation of the fibrocartilage while some histological markers of fibrocartilage can be expressed in the absence of testosterone.

INTRODUCTION

The penis of rodents includes a skeletal structure that is called os penis (baculum). In the rat, os penis is composed of a proximal and a distal segment. The proximal segment of os penis is a Haversian bone with hyaline growth cartilage at its proximal end. The distal segment of os penis is fibrocartilage that gradually becomes ossified after puberty [1–7]. While normal female rats do not have skeletal tissues in the clitoris, bones and cartilages homologous to the os penis can be induced in the clitoris by neonatal treatment with androgen [8–10]. Fetal genital tubercles, in which primordia of the os penis are recognizable in both sexes, can form os penis when transplanted beneath the renal capsule of adult male rats or castrated males that have been treated with androgens [11]. In a previous study, we showed that genital tubercles cultured in vitro can form skeletal elements in the presence of androgens [12]. In addition, the cells of the primordia of os penis acquire androgen-binding capacity during the fetal period [13]. These results indicate that the development of os penis depends on androgens, and that the primordia of os penis are direct targets of androgens. Since the cells that form the skeletal tissues of os penis have distinct cytological characteristics, os penis provides a useful system for studies of cell differentiation caused by androgens. Among the skeletal elements of os penis, the fibrocartilage of the distal segment is a relatively large and non-complex tissue that is composed of chondrocytes and extracellular matrix (ECM). In previous studies, we demonstrated that the ECM of the fibrocartilage of the distal segment contains type I and type II collagens as major components [14], and that the chondrocytes of the fibrocartilage also express strong alkaline phosphatase (ALP) activity, which has been shown to appear in other calcifying tissues, prior to the calcification that occurs after puberty [10]. We also found that the cytoplasm of the chondrocytes is rich in intermediate filaments that react with vimentin-specific antibodies (unpublished data). These histological characteristics provide good markers of the phenotypic differentiation of this fibrocartilage. In order to analyze the role of androgens in the differentiation of the fibrocartilage, primodia of the fibrocartilage were cultured in vitro in the presence and in the absence of testosterone, and the phenotypic expression of various markers was examined.

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MATERIALS AND METHODS

Culture in vitro of the primordium of the distal segment of os penis

Rats of the Wistar Imamichi strain (Imamichi Institute for Animal Reproduction, Oomiya, Japan) were used. The animals were mated during the night and copulation was confirmed the next morning by the presence of spermatozoa in the vaginal smear. The conceptus was designated as being 0.5 days old at noon of this day. Pregnant rats were killed by cervical dislocation and genital tubercles were excised from male and female fetuses. At this stage, a primordium of the distal segment of os penis can be recognized as a mass of mesenchymal cells under a dissection microscope. The genital tubercles were treated with 1 mM EDTA in Tyrode's solution for 15 minutes, and the primordium of the distal segment was isolated with microscissors. The primordia were cultured in vitro essentially as described in a previous report [12]. The isolated explants were laid on a Millipore filter (pore size, 1.2 μm), placed on a stainless-steel grid in a glass dish, and cultured in DMEM supplemented with 20% fetal bovine serum (FBS), 0.2 mM ascorbic acid phosphate, 20 mM β-glycerophosphate, and 10 ng/ml testosterone. In control experiments, no testosterone was added to the medium or cyproterone acetate, an anti-androgen [15], was added at 1 ng/ml to the culture medium to avoid any possible effects of androgens that might be present in FBS. Serum-free medium (ASF301; Ajinomoto, Tokyo, Japan) was also used to avoid potential androgenic effects of the serum. The explants were cultured at 37°C for 7, 10, 12, 14, or 21 days. The culture medium was replaced every three days. The cultured explants were fixed with 95% ethanol for one hour at 4°C, embedded in paraffin (m.p. 46–48°C), sectioned at 4–6 μm and

processed for immunofluorescence or enzyme histochemistry. Some sections were stained with Alcian blue-hematoxylin-eosin for detection of the Alcian blue-stainable ECM that is characteristic of cartilage. To examine calcification of the fibrocartilage, some sections were stained with alizarin red S. Some samples were processed for transmission electron microscopy (TEM) for an examination of calcification.

Immunohistochemical staining specific for type I and type II collagens and vimentin

The paraffin sections were deparaffinized with xylene, rehydrated through a graded ethanol series, washed with PBS and then with water. In order to remove glycosaminoglycans, which might inhibit the binding of antibodies to extracellular collagen fibers, sections were treated with a 0.5% solution of testicular hyaluronidase (Type IS, Sigma, St Louis, MO) in 0.2 M acetate buffer (pH 4.8) for 40 minutes at 37°C. After washing with PBS, adjacent serial sections were incubated either in rabbit antiserum against calf type I collagen (Advance, Tokyo, Japan; diluted 1:40) in PBS, or in rabbit antiserum against calf type II collagen (Advance; diluted 1:150) for one hour at 37°C, then they were washed with PBS and incubated with FITC-conjugated goat antibodies against rabbit IgG (Miles Laboratories, Naperville, IL; diluted 1:64) for one hour at 37°C. Sections were examined with an epifluorescence microscope. For vimentin-specific immunofluorescence, rehydrated sections were incubated in goat antiserun against human vimentin (Sigma; diluted 1:20) for one hour at 37°C, washed in PBS and incubated in FITC-conjugated rabbit antibodies against goat IgG (Sigma; diluted 1:64) for one hour at 37°C.

Histochemical detection of alkaline phosphatase (ALP) activity

The cultured explants were fixed, embedded, sectioned, and deparaffinized as described above. Rehydrated sections were stained for ALP activity by the azo dye method. Naphtol AS-BI phosphate in 0.1 M Tris buffer (pH 9.0) was used as a substrate and Fast red violet L.B. salt was used as the coupling agent. The reaction was allowed to proceed at 37°C for ten minutes.

RESULTS

The numbers of explants examined after each culture period are summarized in Table 1. Since the development of explants taken from male and female fetuses was identical, the following descriptions do not include the sex of the fetuses from which genital tubercles had been removed.

Table 1. Number of explants examined after each period in culture

Medium	Culture period (days)				
	0[1]	7	10	14	21
(+Testosterone)					
DMEM		8	9	27	6
ASF		1	1	4	2
	9				
(Control)					
DMEM		6	6	16	4
DMEM+CA[2]		3			2
ASF		2	1	9	2

1. 20.5-day-old fetuses
2. Cyproterone acetate

Development of fibrocartilage in explants during culture in vitro with testosterone

The primordium of the distal segment of os penis was recognized as a mesenchymal condensation in the genital tubercle of the fetus on day 20.5 of gestation (Fig. 1). At this stage, the cells appeared undifferentiated, with scant extracellular matrix (ECM) around them (Fig. 2). Cells in explants that had been cultured for 7 days with testosterone were larger, and fibrous ECM became apparent (Fig. 3). By day 10, the ECM had increased in volume, and differentiated chondrocytes were visible that were associated with ECM that was stained by Alcian blue (Fig. 4). By day 14, a large number of the cells in the explants had differentiated into mature chondrocytes with a hypertrophic appearance, and the ECM around the chondrocytes was heavily stained with Alcian blue (Fig. 5). In controls cultured without testosterone (DMEM+FBS, DMEM+FBS+cyproterone acetate, or ASF), the cells became enlarged and fibrous ECM also increased in volume during culture. However, the explants remained much smaller than those cultured with testosterone. Cells in the control explants did not differentiate into chondrocytes even after 2 or 3 weeks in culture but formed fibroblastic tissue (Fig. 6). Explants cultured with cyproterone acetate also formed fibroblastic tissue, but explants were very small after 2 weeks of culture. The time courses of development of the explants were consistent among explants after each respective period in culture, and details are summarized in Table 2.

Table 2. Developmental events in explants

Developmental events	Culture period (days)				
	0[1]	7	10	14	21
Fibrous ECM					
+Testosterone	+	+	++	++	++
Control	+	+	+	+	+
Alician blue-stainable ECM					
+Testosterone	−	−	+	++	++
Control	−	−	−	−	−
Chondrocytes					
+Testosterone	−	−	+	++	++
Control	−	−	−	−	−

1. 20.5-day-old fetuses

Expression of type I and type II collagens in explants cultured with testosterone

In the primordium of the distal segment of os penis after 20.5 days of gestation, the fine fibrous extracellular matrix was weakly immunostained with antiserum specific for type I collagen (Fig. 7). Type II collagen was not detected at this stage. After 7 days in culture, the fibrous extracellular matrix of the explants was strongly positive for type I collagen

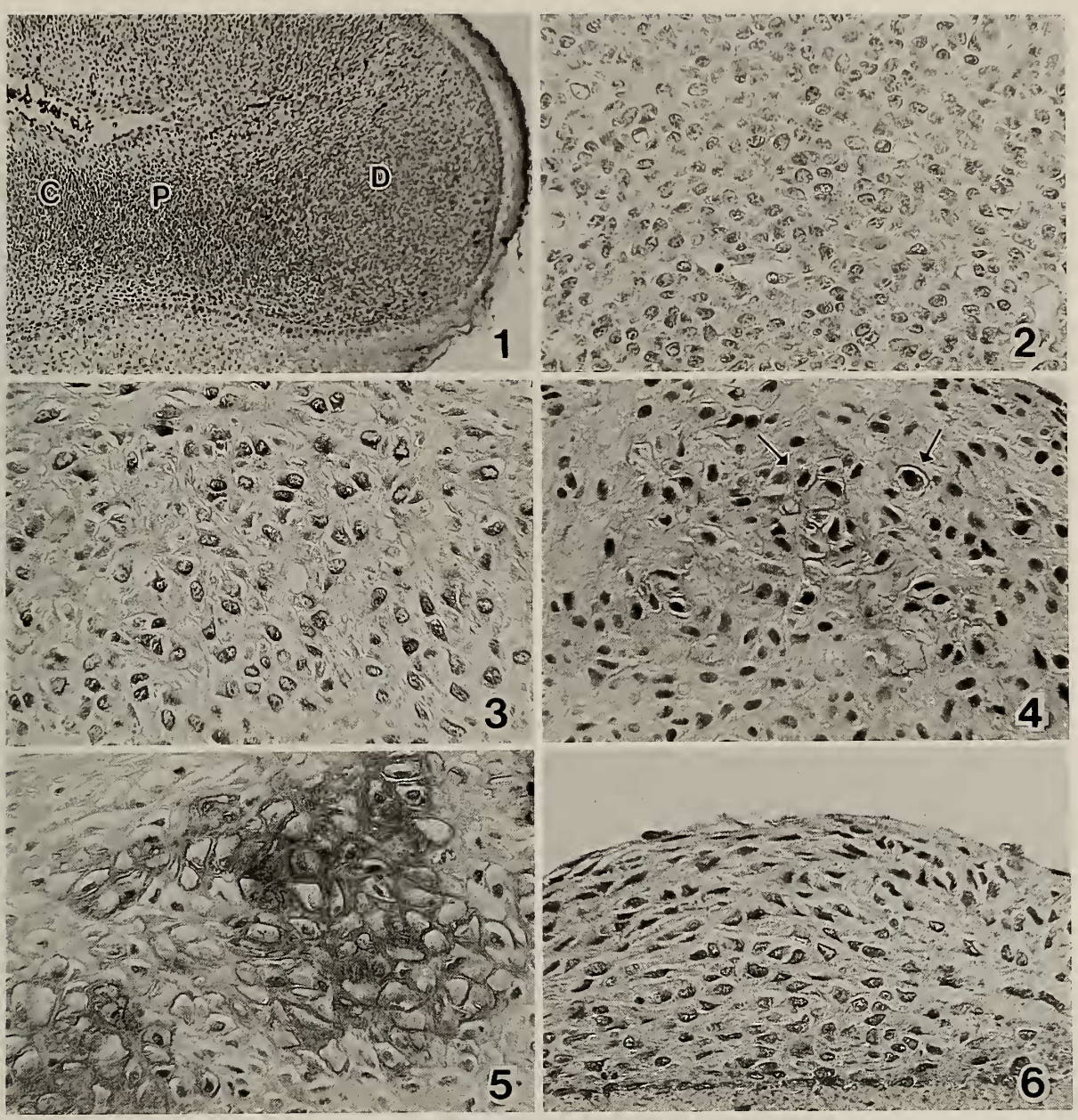

FIG. 1–6. Development of fibrocartilage in explants. Histological sections were stained with Alcian blue-hematoxylin-eosin.

FIG. 1. Longitudinal section of the male genital tubercle of a 20.5-day-old fetus. Primordia of the corpus cavernosum penis (C), proximal segment of os penis (P), and distal segment of os penis are recognizable. 130×.

FIG. 2. Section of the primordium of the fibrocartilage of a 20.5-day-old fetus. Cells are undifferentiated at this stage. 500×.

FIG. 3. Section of an explant after 7 days in culture with testosterone. Cells are large and fibrous ECM is visible. 500×.

FIG. 4. Section of an explant after 10 days in culture with testosterone. Chondrocytes are recognizable (arrows). The ECM around chondrocytes can be stained with Alcian blue. 500×.

FIG. 5. Section of an explant after 14 days in culture with testosterone. A large number of the cells are now mature chondrocytes with a hypertrophic appearance. The ECM around chondrocytes is heavily stained with Alcian blue. 500×.

FIG. 6. Section of a control explant after 14 days in culture. Cells have formed fibroblastic tissue with fibrous ECM. The explant was much smaller than explants that had been similarly cultured with testosterone. 500×.

(Fig. 8). Type II collagen was not detected. On day 10, type I collagen was detected in the ECM throughout the explant, but the fluorescence was diffuse, especially in the inner region of the explant (Fig. 9a). At this stage, chondrocytes appeared in the explants, and type II collagen was detected for the first time in the ECM around clusters of chondrocytes (Fig. 9b). On day 14, a large fraction of the cells in the explants was found to have differentiated into

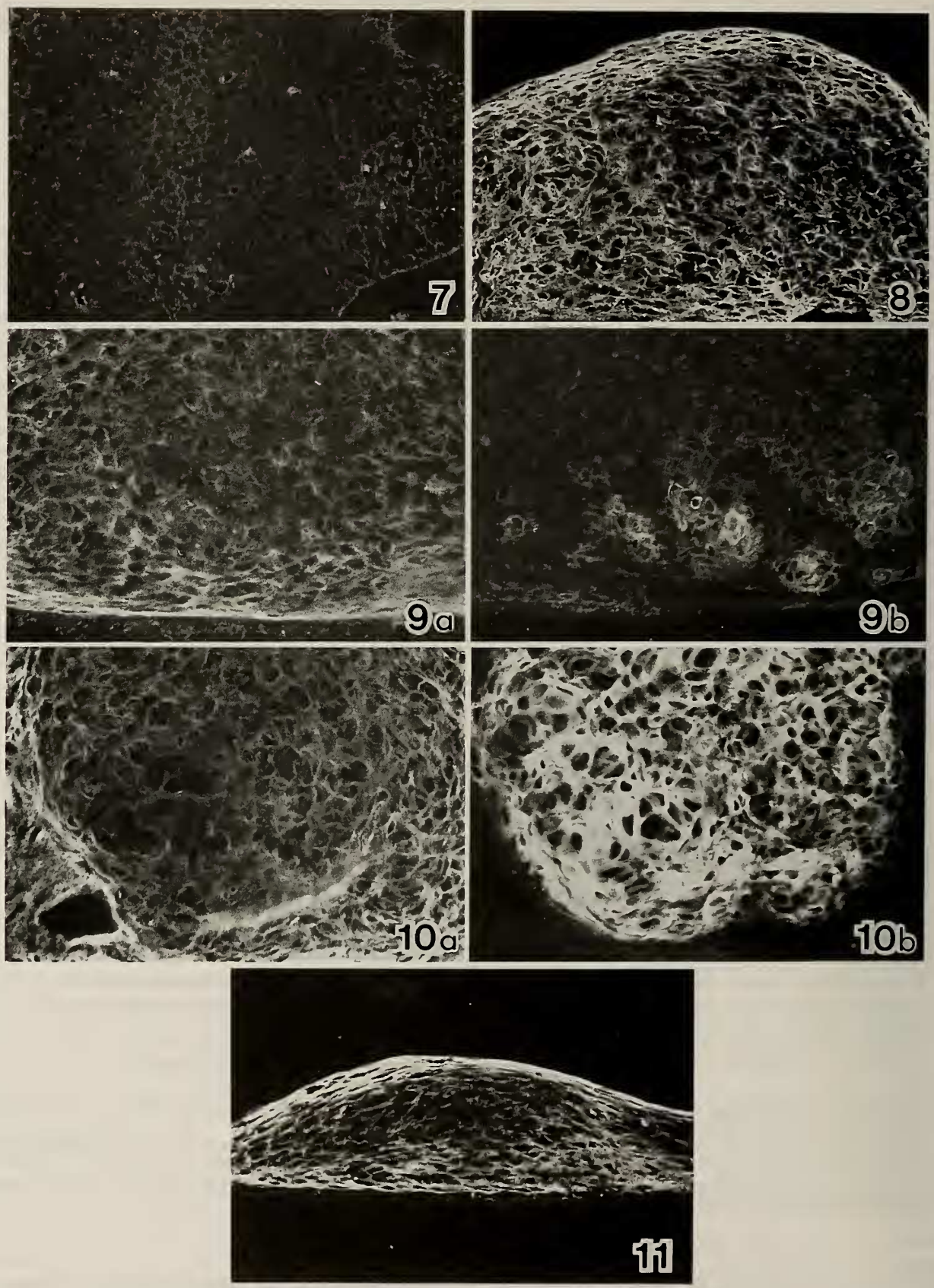

Fig. 7–11. Immunofluorescent staining for type I and type II collagens in explants. 250×.

Fig. 7. Immunofluorescence specific for type I collagen in the primordium of the fibrocartilage from a 20.5-day-old fetus. The fine fibrous ECM is weakly positive. 250×.

Fig. 8. Immunofluorescence specific for type I collagen in an explant after 7 days in culture with testosterone. Fibrous ECM is strongly

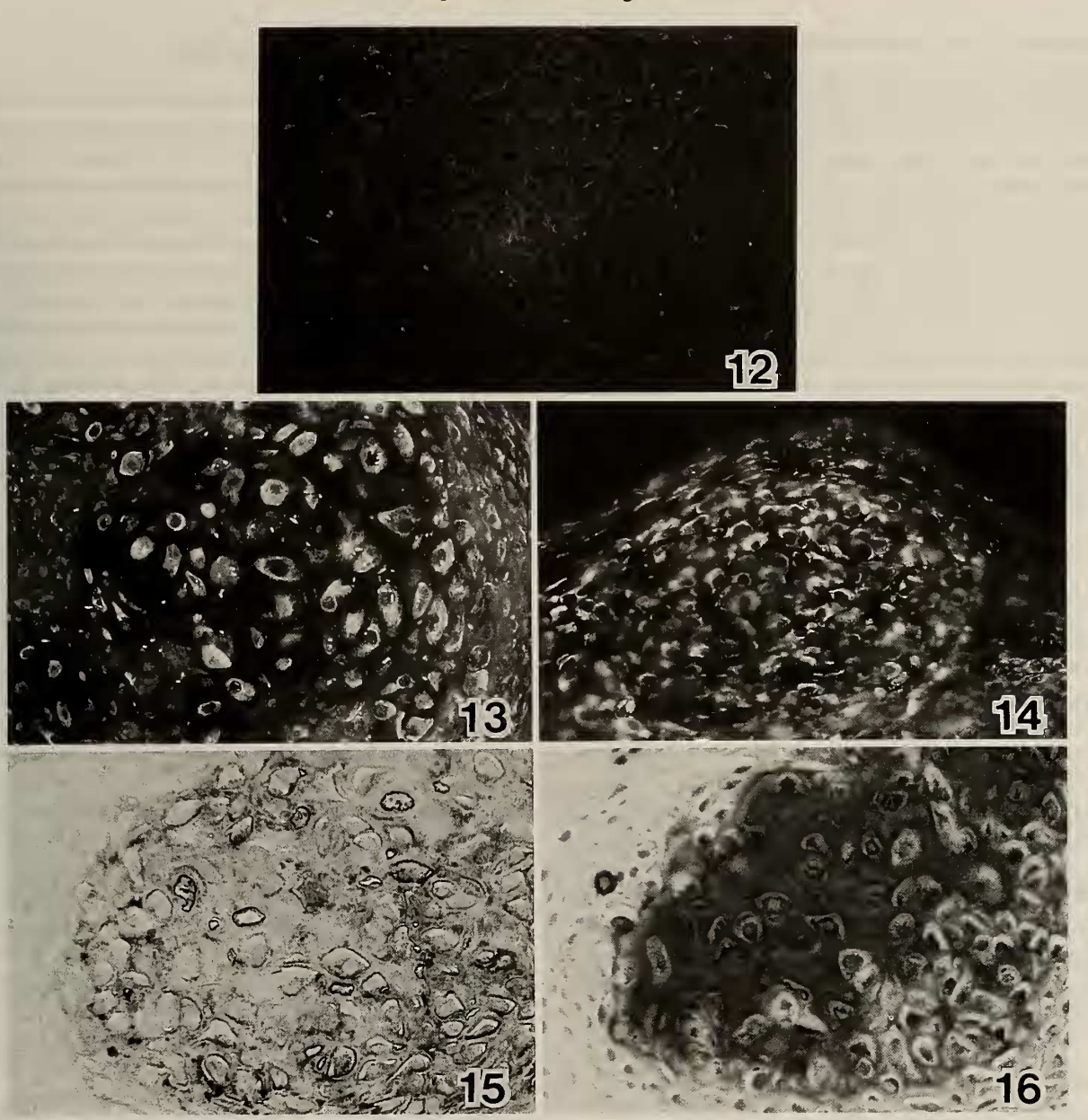

FIG. 12. Immunofluorescence specific for vimentin in the primordium of the fibrocartilage in a 20.5-day-old fetus. The cytoplasm of the cells is weakly immunopositive. 250×.

FIG. 13. Immunofluorescence specific for vimentin in an explant after 14 days in culture with testosterone. The cytoplasm of chondrocytes is strongly positive. 250×.

FIG. 14. Immunofluorescence specific for vimentin in a control explant after 14 days in culture. The cytoplasm of cells is strongly positive. 250×.

FIG. 15. Histochemical detection of ALP activity in an explant after 21 days in culture with testosterone. Chondrocytes are positive for ALP. 250×.

FIG. 16. Staining with alizarin red S for detection of calcification in a section adjacent to that in Fig. 15. The fibrocartilage is heavily calcified. 250×.

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positive. 250×.

FIG. 9a. Immunofluorescence specific for type I collagen in an explant after 10 days in culture with testosterone. Fluorescence from the inner region of the explant is diffuse. 250×.

FIG. 9b. Immunofluorescence specific for type II collagen in a section adjacent to that in Fig. 9a. Small clusters of chondrocytes are positive for immunostaining. 250×.

FIG. 10a. Immunofluorescence specific for type I collagen in an explant after 14 days in culture with testosterone. Immunoreactivity in the inner region of the explant, in which a mass of fibrocartilage has formed, is weaker than in the outer region. 250×.

FIG. 10b. Immunofluorescence specific for type II collagen in a section adjacent to that in Fig. 10a. The ECM of the fibrocartilage is strongly immunostained. 250×.

FIG. 11. Immunofluorescence specific for type I collagen in a control explant after 14 days in culture. The fibrous ECM is strongly positive. 250×.

chondrocytes. The ECM of the explants was positive for type I collagen. However, the immunoreactivity in the inner region of the explants, where most of the cells had become mature chondrocytes with a hypertrophic appearance, was weaker than had been previously observed (Fig. 10a). Staining for type II collagen was intense in the ECM around the mature chondrocytes (Fig. 10b). In the controls that had been cultured without testosterone, fibrous materials in the ECM of the explants were heavily stained for type I collagen after 7 days of culture (Fig. 11), while type II collagen was not detected at any stage. The expression of type I collagen was also detected in explants that had been cultured in serum-free medium (ASF301) or in medium that contained cyproterone acetate.

These results were consistent among explants after each respective period in culture, and details are summarized in Table 3.

Expression of vimentin in explants

Cells in the primordium of the distal segment of a fetus after 20.5 days of gestation were weakly stained with vimentin-specific antibodies (Fig. 12). On day 14 of culture, all the cells in explants cutured with or without testosterone were heavily stained for vimentin (Figs. 13 and 14, Table 3).

ALP activity and calcification

ALP activity first became detectable after 10 days in culture in clusters of chondrocytes in the explants that had been cultured with testosterone. On days 14 and 21, strong ALP activity was detected in the fibrocartilage (Fig. 15). Calcification of the fibrocartilage was observed after 3 weeks in culture (Fig. 16). In controls, neither ALP activity nor calcification was detected (Table 3).

Table 3. Immunohistochemical and histochemical detection of features of differentiation of the fibrocartilage

Feature	Culture period (days)				
	0[1]	7	10	14	21
Type I collagen					
+testosterone	+ (2)	++ (4)	++ (4)	++ (8)	++ (2)
control	+ (2)	++ (4)	++ (2)	++ (6)	++ (1)
Type II collagen					
+testosterone	− (2)	− (4)	+ (4)	++ (8)	++ (2)
control	− (2)	− (4)	− (2)	− (6)	− (1)
Vimentin					
+testosterone	+ (2)			++ (4)	
control	+ (2)			++ (3)	
ALP					
+testosterone		− (1)	+ (2)	++ (2)	++ (2)
control				− (2)	− (1)
Calcification					
+testosterone		− (3)	− (5)	− (7)[2]	+ (4)
control		− (2)	− (2)	− (4)	− (3)

( ): Numbers of explants examined
1. 20.5-day-old fetuses
2. Small electron-dense particles were observed

DISCUSSION

Sexual dimorphism in mammalian external genitalia is determined, for the most part, by the hormonal secretions of the testis: androgens induce the development of the male phenotype, and the absence of gonads results in the female phenotype [16]. Mesenchymal cells have been shown to be essential for the development of sexual dimorphism in accessory sex organs, and they have been shown to be primary targets of androgens in developing prostatic glands [17, 18], mammary glands [19], the prepuce, and the penis [13]. However, responses of the mesenchymal cells to androgens and subsequent processes of cell differentiation have not previously been investigated in detail because most of the mesenchymal cells do not have distinctive histological characteristics that can be used as markers of cell differentiation. The stimulatory effects of androgens on the proliferation of cells in epiphyseal cartilage have been reported [20], but effects on undifferentiated precursor cells of cartilage have not been studied. Os penis of the rat provides a useful system with which cellular responses to androgens can easily be investigated because it has distinct histological and cytological markers and its development has been characterized in detail [10–14]. In the present study, it was found that explants of the primordium of the distal segment of os penis, when cultured in vitro, formed fully mature fibrocartilage in the presence of testosterone. Calcification of the fibrocartilage was also observed. Results obtained in the present study clearly demonstrate the essential role of the androgen in the differentiation of the fibrocartilage in the penis. The explants expressed histological markers characteristic of the fibrocartilage of os penis, namely, type I and type II collagens, Alcian blue-stainable ECM, vimentin, and ALP. Control explants cultured without testosterone formed fibroblastic tissue with fibrous ECM that contained type I collagen, and the cells in the control explants expressed vimentin, even though the latter explants were smaller than those cultured with testosterone. No type II collagen or ALP activity was detected in controls. These results demonstrate that the expression of type I collagen and vimentin does not depend on testosterone, while the expression of type II collagen, Alcian blue-stainable ECM, and ALP depends on exposure to testosterone (Table 4). Since the reactivity specific for type I collagen became weaker in the regions in which mature chondrocytes appeared, it is possible that the synthesis of type I collagen is suppressed in mature chondrocytes.

Table 4. Dependence on testosterone of the development of various features of the differentiation of the fibrocartilage

Feature	+Testosterone	Control
Type I collagen	+	+
Type II collagen	+	−
Vimentin	+	+
ALP	+	−
Calcification	+	−

In our experiments, the responses to testosterone differed among the various markers, suggesting that the effect of testosterone is a qualitative one, namely, the induction of the terminal differentiation of the fibrocartilage, rather than a general anabolic effect. The cytological markers analyzed here all represent the end products of the translation of genes. Hence, the results obtained do not necessarily reflect the direct activation of specific genes by testosterone. If we are to clarify the molecular actions of testosterone that lead to the differentiation of the fibrocartilage of os penis, it is important that we determine whether testosterone (or its metabolite, dihydrotestosterone) directly activates transcription of the genes for type II collagen and ALP. The system for culture in vitro of the fibrocartilage of os penis described herein should be useful for studies of the differentiation of cells that is caused by androgens.

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